ELSEVIER

Atherosclerosis 192 (2007) 266–274

ATHEROSCLEROSIS

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Common variants of apolipoprotein A-IV differ in their ability to inhibit low density lipoprotein oxidation

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Received 19 December 2005; received in revised form 29 June 2006; accepted 14 July 2006
Available online 1 September 2006

Abstract

Apolipoprotein A-IV (apoA-IV) inhibits lipid peroxidation, thus demonstrating potential anti-atherogenic properties. The aim of this study was to investigate how the inhibition of low density lipoprotein (LDL) oxidation was influenced by common apoA-IV isoforms. Recombinant wild type apoA-IV (100 μg/ml) significantly inhibited the oxidation of LDL (50 μg protein/ml) by 5 μM $CuSO_4$ ($P < 0.005$), but not by 100 μM $CuSO_4$, suggesting that it may act by binding copper ions. ApoA-IV also inhibited the oxidation of LDL by the water-soluble free-radical generator 2,2′-azobis(amidinopropane) dihydrochloride (AAPH; 1 mM), as shown by the two-fold increase in the time for half maximal conjugated diene formation ($T_{1/2}$; $P < 0.05$) suggesting it can also scavenge free radicals in the aqueous phase. Compared to wild type apoA-IV, apoA-IV-S347 decreased $T_{1/2}$ by 15% ($P = 0.036$) and apoA-IV-H360 increased $T_{1/2}$ by 18% ($P = 0.046$). All apoA-IV isoforms increased the relative electrophoretic mobility of native LDL, suggesting apoA-IV can bind to LDL and acts as a site-specific antioxidant. The reduced inhibition of LDL oxidation by apoA-IV-S347 compared to wild type apoA-IV may account for the previous association of the *APOA4* S347 variant with increased CHD risk and oxidative stress.

Keywords: Atherosclerosis; apoA-IV; Conjugated dienes; Copper; Lipid hydroperoxides; Oxidised low density lipoprotein

1. Introduction

According to the oxidative modification hypothesis of atherosclerosis, the oxidation of low density lipoprotein (LDL) is a major step in the progression of atherogenesis [1]. Oxidised LDL (oxLDL) can contribute to atherogenesis via a variety of mechanisms, for instance, it is internalised rapidly by macrophages via their scavenger receptors, leading foam cell formation [2], is chemotactic for monocytes [3] and can stimulate the binding of monocytes to the endothelium [4]. OxLDL is also cytotoxic to cells in the vasculature [5]. Despite the disappointing effects of antioxidants in humans to significantly reduce coronary heart disease (CHD) [6,7], animal studies have suggested lipid soluble antioxidants, even though used at far greater physiological concentration, can sometimes reduce lesion formation independently of plasma lipid concentrations [8].

Apolipoprotein A-IV (apoA-IV) is a 46-kDa glycoprotein, which in humans is synthesised by enterocytes of the small intestine [9]. ApoA-IV is secreted into the lymph bound to chylomicrons and is rapidly displaced by the apolipoprotein Cs (apoCs) [10,11]. Using a non-disruptive separation technique it has been shown that apoA-IV is present in three fractions; lipid-free apoA-IV representing about 4%

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doi:10.1016/j.atherosclerosis.2006.07.017

of total apoA-IV, a fraction associated with apoA-I (LpA-I:A-IV, 12%), an apoA-I-free, lipoprotein fraction (LpA-IV, 84%) [12]. *In vitro* apoA-IV has been shown to activate the enzymes lecithin:cholesterol acyl transferase [13,14], facilitate the action of cholesterol ester transfer protein [15], and modulate lipoprotein lipase activity [16]. Collectively these actions could promote the transport of cholesterol from extra-hepatic tissue and cells to the liver [17,18]. However it is presently unclear whether these are true *in vivo* physiological functions of apoA-IV.

In addition to these classical roles in lipid metabolism, studies have demonstrated that apoA-IV possesses anti-atherogenic properties. Over expression of *APOA4* in transgenic *Apoe−/−* mice conferred protection against diet-induced atherosclerosis, independent of HDL concentration [19]. In case:control studies, patients with coronary artery disease (CAD) had lower apoA-IV plasma levels compared to healthy subjects [20,21]. Although the anti-atherogenic properties of apoA-IV could be the consequence of its ability to promote reverse cholesterol transport, as described above, several lines of evidence suggest that it could also result from its ability to act as an anti-oxidant. Firstly, *APOA4*, when expressed in *Apoe−/−* mice reduced oxidative markers, such as anti-oxLDL antibodies and 4-hydroxy-2,3-transnonenal (4HNE) modified LDL [22]. Secondly, human apoA-IV *in vitro* inhibits the lipid peroxidation of VLDL by both copper ions (Cu^{2+}) and 2,2-azobis-[2-amidinopropane dihydrochloride] (AAPH) [23]. Recently expression of *APOA4* in *Apoe−/−* mice treated with lipopolysaccharide as a model of chronic infection, significantly reduced the size of atherosclerotic lesions and the secretion of proinflammatory cytokines, compared to control *Apoe−/−* littermates, despite an increased oxLDL concentrations [24]. Similarly, injection of recombinant apoA-IV into mice significantly reduced the inflammatory effects of dextran sulphate sodium, and the P-selectin-induced recruitment of leukocytes [25].

ApoA-IV displays several common genetic polymorphisms. The most common polymorphism worldwide is an ACT > TCT substitution at codon 347, with an allele frequency of 0.20–0.25, that encodes a serine for threonine substitution (T347S) and yields the apoA-IV-1A isoform [26]. In Caucasian populations the next most common polymorphism is a CAG > CAT substitution at codon 360, with an allele frequency of 0.08–0.12, that encodes a histidine for glutamine substitution (Q360H), yielding the more basic apoA-IV-2 isoform. Finally, an AAC > AGC substitution in codon 127, encodes an asparagines to serine substitution (N127S) and produces the apoA-IV-1B isoform [27].

In recent studies we have demonstrated an association of the *APOA4* S347 variant with an increased risk of CHD, independent of effects on plasma lipids [28], and significantly lower apoA-IV plasma levels and reduced total antioxidant status (TAOS) in diabetic subjects with cardiovascular disease (CVD) [29]. To investigate whether the increased CHD risk in S347 homozygotes could be due to reduced anti-oxidant activity of the apoA-IV-S347 isoform, we investigated the effect of the common apoA-IV isoforms on conjugated diene formation in LDL oxidised with both copper and AAPH.

2. Materials and methods

2.1. Materials

All chemicals used were of AR grade or better. $CuSO_4$, 2,2′-azobis(amidinopropane) dihydrochloride (AAPH) and Chelex-100® were obtained from Sigma, UK. Phosphate buffer saline (PBS) and bovine serum albumin (BSA) were manufactured by Gibco and Firstlink (UK), respectively.

2.2. Mutagenesis of APOA4

The human *APOA4* cDNA was inserted between the *Nde*I and *Bam*HI sites of the expression vector pET-14b vector containing a 6× N-terminal histidine tag sequence (his-tag) supplied by Novagen (Nottingham, UK). Such constructs were subjected to site directed mutagenesis using the QuikChange kit™ (Stratagene, Cedar Creek, USA) to produce the appropriate *APOA4* variants. Mutagenesis was carried out according to the manufacturer's instructions with two synthetic oligo primers (Invitrogen, Paisley, UK) containing the desired mutation and complementary to opposite strands of the vector. The sequence for the mutagenic primers with the base changes in bold are:

- T347S
  - forward: 5′GCCAGGACAAG**T**CTCTCTCCCTCCC3′
  - reverse: 5′GGGAGGGAGAGAG**A**CTTGTCCTGGC3′
- Q360H
  - forward: 5′GCAGGAACAGCA**T**CAGGAGCAGCAGC3′
  - reverse: 5′GCTGCTGCTCCTG**A**TGCTGTTCCTGC3′
- N127S
  - forward: 5′GCGCACCCAGGTCA**G**CACGCAGGCCGAGC3′
  - reverse: 5′GCTCGGCCTGCGTG**C**TGACCTGGGAGCGC3′.

All constructs were confirmed by sequencing, using an ABI 377 sequencer and Bigdye™ terminator V3.1 sequencing kit from Applera (Norwalk, USA).

2.3. Production and purification of recombinant apoA-IV

An expression system under the control of the T7 promoter for the production of his-tagged apoA-IV proteins, ready for affinity tag purification, was used. The pET-14b expression vectors containing wild type *APOA4* or *APOA4* variant inserts were individually transformed into *Escherichia coli* BL21 (DE3) bacteria. The transformed bacteria were grown in a shaker flask overnight at 37 °C in Luria broth medium, sup-